Combined Enzymic–Jaffé Method for Determination of Creatinine in Serum

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Concentrations of creatinine, as determined in serum by a method involving the combined use of creatinine amidohydrolase (EC 3.5.2.10) and alkaline sodium picrate were found to be factitiously low, owing to a reversal of the enzyme reaction. This effect could be eliminated by converting creatine, the product of the enzymic reaction, to creatine phosphate. The combined enzymic–Jaffé method was therefore modified to include creatinine amidohydrolase, creatine kinase, ATP, and Mg²⁺ in the reaction mixture. The modified method has good precision. We saw no significant interferences by relatively high concentrations of acetone, acetylacetone, ADP, creatine, creatine phosphate, glucose, glycocolline, or pyruvate. Likewise, no interferences were evident with icteric, lipemic, or hemolytic serum samples. There was an excellent agreement between creatinine values obtained with our method and by a reference method based on isotope dilution–mass fragmentography. Our method is considerably simpler than the fully enzymic method for determination of creatinine and might be a method of choice if a high accuracy is desired.

The most commonly used method for the quantitative determination of creatinine is based on the Jaffé reaction, involving alkaline sodium picrate (1). However, this reaction is affected by more than 50 endogenous compounds (2). Several modifications of the Jaffé reaction have been devised in an attempt to eliminate or diminish the interference by these pseudocreatinines. These include specific adsorption of creatinine (3, 4), extraction of the interfering compounds (4), dialysis (5), use of different pH (6, 7), and kinetic measurements (8–10). Unfortunately, none of these modifications eliminates all the interferences from the large number of pseudocreatinines present in serum in various concentrations and possessing differing properties. Fully enzymic methods have recently been introduced to improve the specificity of the determination (11–13). Although these methods appear to give more accurate results when used under optimum conditions (14), they are laborious and have a relatively low degree of precision (15). Moreover, the use of a multi-enzyme system requires caution, because the risk for interferences with the enzymes increases with the increasing number of the enzymes used.

The use of creatinase in combination with the Jaffé reaction was reported by Miller and Dubos (16). However, the enzyme used was a crude preparation, which actually contained a mixture of creatinase (creatinine amidohydrolase, EC 3.5.2.10), which catalyzes the conversion of creatinine to creatine, and creatinase (creatinine amidohydrolase, EC 3.5.3.3, which catalyzes the hydrolysis of creatine to sarcosine and urea). When we combined the use of purified creatinine amidohydrolase with the Jaffé reaction, the creatinine concentrations we obtained were falsely low. In this report, we show that this discrepancy is caused by creatine, and that the effect can be eliminated by converting creatine to creatine phosphate. We also present an evaluation of this method.

Materials and Methods

Reagents

The following chemicals were of analytical grade unless specified otherwise: acetone, acetylacetone, creatine, creatinine, D-glucose, glycoglycerine, magnesium chloride, pyruvate (Merck), Triton X-100 surfactant (scintillation grade, Eastman Kodak), adenosine-5-diphosphate disodium salt (ADP), adenosine-5-triphosphate disodium salt (ATP), creatinase (from Alcaligenes species, in 500 mL/L glycerol, activity about 350 kU/L), creatine kinase (CK; ATP:creatine N-phosphotransferase, EC 2.7.3.2; from rabbit muscle, activity about 25 kU/g of lyophilizate), disodium creatine phosphate (Boehringer Mannheim), and guanidinoacetic acid (glycocynamine, Sigma Chemical Co.). A solution containing 57 mmol of picric acid per litre, adjusted to pH 8.0 with 5 mol/L NaOH, was obtained from a local distributor.

Alkaline Picrate Solution

The picric acid reagent was diluted fivefold with a solution containing 500 mmol of NaOH per litre, and 2.5 mL of Triton X-100 was added per litre of reagent. The final pH was 12.8.

Choice of Buffer and pH

The buffer system described by Moss et al. (13) was used with minor modifications, and contained 75 mmol of glycoglycerine and 2.5 mL of Triton X-100 per litre. The pH was adjusted to 8.8 with 2 mol/L NaOH.

General Method with Creatinine Amidohydrolase

This involved a pre-incubation of 100 μL of sample and 100 μL of the enzyme (about 5000 U/L) in buffer. The enzyme was omitted from the buffer solution used for the sample blank. Alkaline picrate solution, 800 μL, was then added to both the sample and its blank, and the difference in absorbance (ΔA) between the two samples measured at 510 nm with an LKB 7400 spectrophotometer (LKB Produkter, Sweden). The concentration of creatinine was determined by comparison of ΔA values for the samples and standard solutions. The optimum conditions for the method were determined as described in Results.

Results

Analytical Variables

Kinetics of the Jaffé reaction. Absorbances at 510 nm were monitored for 45 min after alkaline picrate at 25 °C was added to several patients' samples and to standard solutions. The reaction with the standards stabilized within 10 min, but proceeded continuously with the patients' samples. However, ΔA, the difference in absorbance between the sample (with enzyme) and the sample blank (without enzyme) remained constant after 5 min. We used an incubation time of 15 min in all subsequent work.

Effect of creatinine amidohydrolase. Standard solutions containing 100 and 200 μmol of creatinine per litre were an-

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analyzed by the general method at 25, 30, and 37°C. Because ΔA values at 25°C were higher than those at the other temperatures, we used a temperature of 25°C.

The same standards were used to determine the optimum amount of creatinine amidohydrolase required for the conversion of creatinine. In addition to the sample and its blank, we also analyzed a reagent blank for alkaline picrate. The difference in absorbance between the sample blank and the reagent blank (ΔA1) was due to all the creatinine present and represented ΔA for a 100% Jaffé reaction. Similarly, the difference in absorbance between the sample and the reagent blank (ΔA2) was due to the residual creatinine present after the enzymic reaction. Thus, ΔA1 − ΔA2 represented the amount of creatinine converted by the enzyme. We observed a conversion of 60% with a final enzyme activity of 3 kU/L. Increasing the enzyme activity to 14 kU/L improved the conversion only slightly, to 68%.

In a preliminary study, the creatinine content in 20 patients' samples was determined by creatinine amidohydrolase–Jaffé method and by a fully enzymic method (Test Combination, creatinine enzymatic, Boehringer Mannheim). In general, creatinine values as measured by the enzymic–Jaffé method were 30 to 70 μmol/L lower than corresponding values with the fully enzymic method. Several negative results were also obtained. These findings necessitated further investigations of the enzymic–Jaffé method.

Effects of creatine. To study the effects of creatine on the creatinine amidohydrolase–Jaffé method, we analyzed solutions containing a mixture of creatinine (100 μmol/L) and increasing concentrations of creatine. ΔA values were observed to decrease with increasing concentrations of creatine, leading to negative values at higher concentrations (Figure 1). Identical results were also obtained with solutions containing only creatine; however, when these ΔA values were subtracted from the corresponding ΔA values obtained in the presence of creatinine, the resulting analytical recoveries for creatinine were found to be excellent (Table 1). These data illustrate that creatine does not inhibit creatinine amidohydrolase, but that a reaction between the two produces a Jaffé-positive chromogen.

To determine the nature of this chromogen, we incubated 1.0 mL of a solution containing 250 or 500 μmol of creatine per litre of glycylglycine buffer, pH 8.8, at 25 or 37°C for 10 min with creatinine amidohydrolase (final activity of 5200 U/L).

The samples were then boiled for 6 min to denature the enzyme. Concurrently, we treated control samples with heat-denatured enzyme. Finally, the creatinine content in all the samples was determined with the fully enzymic method. We found that up to 30% of the creatine was converted by creatinine amidohydrolase to creatinine at 25°C and up to 47% at 37°C. No conversion was observed with heat-denatured enzyme, demonstrating that the production of creatinine was a result of the reversal of the enzymic reaction.

We also assessed the effect of creatine phosphate by performing recovery studies similar to those for creatine, and found that it did not affect the recoveries significantly. Thus, we believed that the negative effect of creatine could be eliminated if the reaction was extended towards the production of creatine phosphate.

Effects of CK, ATP, and Mg2+. We used a mixed standard solution containing 100 μmol of creatinine and 200 μmol of creatine per litre to determine the optimum conditions for the conversion of creatine. The method was as follows: 100 μL of the sample was incubated at 25°C for 5 min with 50 μL of a buffered solution containing CK, ATP, and Mg2+. Creatinine amidohydrolase (50 μL of a 10.4 kU/L solution) was then added and the incubation continued for 10 min. The buffer solution without the enzymes or ATP was used for the blanks. The rest of the procedure, with alkaline picrate, was as outlined before.

The equilibrium of the conversion of creatine to creatinine phosphate depends on several factors, such as pH, concentrations of ATP, and Mg2+, and the activity of CK. The forward reaction is favored by pH values between 8.6 and 8.8. Increasing concentrations of creatine were obtained with increasing concentrations of ATP, the maximum effect occurring at a concentration of 5 mmol/L. Similarly, the most effective concentration of Mg2+ was about 0.4 mmol/L. Figure 2 shows some of the interrelationships between the effects of ATP, Mg2+, and CK. Evidently, a relatively low activity of CK is required for the forward reaction.

The effect of creatinine amidohydrolase was re-examined in the presence of the optimized auxiliary system. A conversion of 95% was achieved with 2600 U of creatinine amidohydrolase per litre in combination with CK, compared with 58% in the absence of CK. Higher activities of creatinine amidohydrolase did not increase the percentage conversion significantly. A reaction time of 5 min was sufficient for each enzyme.

Evaluation of the Combined Enzymic–Jaffé Method

The protocol for the finally adopted method is shown in Table 2. The glycylglycine buffer was reconstituted to include Mg2+. All solutions were stable for as long as two weeks at 8°C.

Linearly. Seven aqueous samples ranging in concentration from 25 to 1000 μmol of creatinine per litre were analyzed in triplicate on six different occasions. Linear regression statistics

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**Table 1. Analytical Recovery of Creatinine in the Presence of Creatine**

<table>
<thead>
<tr>
<th>Creatine, μmol/L</th>
<th>Creatinine added, μmol/L</th>
<th>ΔA1</th>
<th>ΔA2</th>
<th>ΔA = ΔA1 - ΔA2</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.025</td>
<td>0.025</td>
<td>0.025</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>-0.006</td>
<td>0.020</td>
<td>0.026</td>
<td>0.026</td>
<td>104</td>
</tr>
<tr>
<td>100</td>
<td>-0.013</td>
<td>0.013</td>
<td>0.026</td>
<td>0.026</td>
<td>104</td>
</tr>
<tr>
<td>200</td>
<td>-0.026</td>
<td>0.00</td>
<td>0.026</td>
<td>0.026</td>
<td>104</td>
</tr>
<tr>
<td>400</td>
<td>-0.052</td>
<td>-0.027</td>
<td>0.025</td>
<td>0.025</td>
<td>100</td>
</tr>
</tbody>
</table>

\( \Delta A = A_{\text{sample blank}} - A_{\text{sample}} \)
were used to show that the results by this method were linear up to a concentration of 500 μmol of creatinine per litre. The regression equation for expected (x) vs observed (y) creatinine concentration was y = 1.01x - 0.87 (r = 0.9996). The standard errors for the slope and intercept were 0.005 and 1.29, respectively. The linearity was also tested with two different serum pools where creatinine was added. The regression equation up to 500 μmol of creatinine per litre was y = 0.97x + 4.88 (r = 0.9996). The standard errors of the slope and intercept were 0.008 and 2.16, respectively.

Because endogenous compounds and proteins in serum also react with the alkaline picrate, we recommend the use of serum standards for method calibration.

Precision. Two commercial sera (Hyland) and two different pools of laboratory samples were used for serial analyses and for between-day studies. Patients’ samples were used for within-day studies. As seen from Table 3, the coefficients of variation for serial analyses were between 2 and 7%, depending on the concentration of creatinine. Within-day precision and between-day precision were also acceptable.

Recovery. We added different amounts of creatinine to 10 patients’ samples and to three different serum pools containing, per litre, 350 μmol of bilirubin, 9 mmol of triglycerides, or 1.5 g of hemoglobin. Between 93 and 104% of the added creatinine was accounted for in each case. The specificity of the method was checked further by analytical recovery studies performed after addition of acetone (1–5 mmol/L), acetylace- tone (1–5 mmol/L), ADP (0.5–15 mmol/L), creatine (25–300 μmol/L), creatine phosphate (50–300 μmol/L), glucose (2.5–60 mmol/L), glycocarnine (50–400 μmol/L), or pyruvate (0.25–2 mmol/L) to pooled serum samples containing between 25 and 200 μmol of creatinine per litre. The recoveries in all cases ranged from 95 to 105%.

Because creatine phosphate is the end product of the combined enzymic reactions, we thought it interesting to see whether endogenous alkaline phosphatase interfered by converting the creatine phosphate back to creatine. To test this, we added 200 μmol of creatinine and 300 μmol of creatine phosphate per litre of serum (final concentration) to five patients’ samples, in which alkaline phosphatase activities ranged from 130 to 3300 U/L. The recoveries for creatinine were between 95 and 99%, suggesting that the risk of interference caused by alkaline phosphatase was negligible.

Comparison with a reference method. Creatinine concentrations in 44 patients’ samples were determined by the combined enzymic–Jaffé method (y) and by isotope dilution–mass fragmentography (x) (17). The regression equation for the comparison was y = 1.03x - 2.85 (r = 0.99) for creatinine concentrations ranging from 39 to 230 μmol/L. The standard error for the slope was 0.025, and the t-test showed

Table 2. Protocol for the Present Method

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample blank vol. μL</th>
<th>Sample vol. μL</th>
<th>Concentrations in the reaction mixture, a per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>100</td>
<td>100</td>
<td>Creatinine, 0–250 μmol</td>
</tr>
<tr>
<td>Glycylglycine</td>
<td>100</td>
<td>100</td>
<td>Glycylglycine, 38 mmol; Triton</td>
</tr>
<tr>
<td>buffer, pH 8.8</td>
<td></td>
<td></td>
<td>X-100, 1.25 mL; Mg2+, 375 μmol</td>
</tr>
<tr>
<td>CK, ATP in buffer</td>
<td>—</td>
<td>50</td>
<td>CK, 12 500 U; ATP, 5 mmol</td>
</tr>
<tr>
<td>Mix and incubate</td>
<td></td>
<td></td>
<td>for 5 min at 25 °C</td>
</tr>
<tr>
<td>CK amidehydrolase</td>
<td>50</td>
<td></td>
<td>Creatinine amidohydrolase,</td>
</tr>
<tr>
<td>in buffer</td>
<td></td>
<td></td>
<td>2600 U</td>
</tr>
<tr>
<td>Mix and incubate</td>
<td></td>
<td></td>
<td>for 10 min at 25 °C</td>
</tr>
<tr>
<td>Alkaline picrate</td>
<td>800</td>
<td>800</td>
<td>Picrate, 9.12 mmol; NaOH, 320 mmol</td>
</tr>
<tr>
<td>Mix and incubate</td>
<td></td>
<td></td>
<td>for 15 min at 25 °C</td>
</tr>
<tr>
<td>Measure absorbance</td>
<td></td>
<td></td>
<td>at 510 nm</td>
</tr>
<tr>
<td>Creatinine concn,</td>
<td></td>
<td></td>
<td>μmol/L = [ΔA (test)/ΔA (standard)] × concn</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(standard)</td>
</tr>
</tbody>
</table>
| a The concentrations of the components during the preincubation phase are calculated for a reaction volume of 200 μL.
| b To avoid turbidity in the buffered reagent, CK and ATP should be dissolved separately before they are mixed.

Table 3. Precision Studies

<table>
<thead>
<tr>
<th></th>
<th>Within-run</th>
<th>Within-day</th>
<th>Between-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>x, μmol/L</td>
<td>48.50</td>
<td>90.20</td>
<td>377.20</td>
</tr>
<tr>
<td>SD, μmol/L</td>
<td>3.33</td>
<td>4.02</td>
<td>5.65</td>
</tr>
<tr>
<td>CV, %</td>
<td>6.9</td>
<td>4.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>
that the slope of 1.03 was not significantly different from 1.00 \( (p > 0.1) \). The standard error of the intercept was 2.42, and the \( t \)-test showed that the intercept value was not significantly different from 0 \( (p > 0.1) \). Statistical analysis by the paired \( t \)-test \( (t = -0.236) \) confirmed that there was an excellent agreement between these two methods. The same 44 samples were also analyzed by a kinetic Jaffé method \( (y) \). In this case, the regression equation was \( y = 1.04x + 33.75 \) \( (r = 0.88) \). Statistical analysis by the paired \( t \)-test \( (t = -11.06) \) confirmed that there was a significant difference in the results obtained by the two methods. This accords with a recent report \( (18) \).

**Discussion**

The concept of combining enzymic and chemical reactions has several advantages. Firstly, the use of an enzyme specific for the substrate increases the specificity of the method. Secondly, the simultaneous use of an appropriate sample blank compensates for the nonspecific interferences on the chemical reaction. The use of creatinine amidohydrolase in combination with the alkaline pirocat reagent is one such example, and has been reported previously \( (16) \); however, in that approach it was assumed that creatinine amidohydrolase is absolutely specific for creatinine. Our studies illustrate that this is not the case: the enzyme also acts upon creatine (endogenous or generated) to produce creatinine. As a result, the \( \Delta A \) values in the ensuing Jaffé reaction were falsely decreased, leading to the decrease in the apparent creatinine concentrations. This effect is more pronounced at 37 °C than at 25 °C.

The negative effect of creatine could be eliminated by converting it to creatine phosphate. We found that this reaction is very sensitive to pH, activity of CK, and concentrations of ATP and Mg\(^{2+} \). A pH value between 8.6 and 8.8 favors the forward reaction. A low activity of CK, a high concentration of ATP, and a low concentration of Mg\(^{2+} \) are required to achieve the optimum effect. These analytical conditions differ from those advocated for the fully enzymic methods \( (11, 13) \).

Our evaluation shows that our method has good specificity. The method has good precision at normal and high concentrations of creatinine. The precision was lower (CV about 7%) at low concentrations of creatinine, and attempts to improve this are in progress. However, we currently regard this precision as acceptable because of the clinical insignificance of low creatinine values. Creatinine concentrations obtained by our method and by isotope dilution–mass fragmentography agreed well. Considering that previous comparisons between several routine methods and the reference method have been unsatisfactory \( (17) \), our method should clearly be preferred if accuracy is the most important criterion. The accuracy of the combined method equals or exceeds that of the fully enzymic method, but our method is simpler, cheaper, and probably more precise. It has also been tested with a few urine samples and appears to be satisfactory.

**References**